PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Markus HECKER & Andreas H. WAGNER	Group Art Unit: 1632
Serial No.: 10/526,430	Examiner: D. Montanari
Filed: March 1, 2005	Atty. Dkt. No.: DEBE:052US/SLH
For: PHARMACEUTICAL FORMULATION WITH NONSTEROIDAL ANTIPHLOGISTICS AND NUCLEIC ACIDS FOR TRANSFERRING NULCEIC ACIDS INTO EUKARYOTIC CELLS	Confirmation No. 9671

CERTIFICATE OF ELECTRONIC TRANSMISSION
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May 28, 2009
Date

Steven L. Highlander

APPEAL BRIEF

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APPEAL BRIEF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-01450

Dear Sir:

This Appeal Brief is filed in response to the Office Action mailed on November 28, 2008. Appellant's brief is due on July 28, 2009, by virtue of the Notice of Appeal filed on May 28, 2009. Also included herewith is the fee for the brief. No other fees are believed due in connection with this filing; however, should appellants' payment be missing or deficient, or

should any fees be due, the Commissioner is authorized to debit Fulbright & Jaworski L.L.P. Deposit Acct. No. 50-1212/DEBE:052US/SLH.

I. Real Party In Interest

The real party in interest is the assignee, Avontec Gmbh, Göttingen, GERMANY.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of the Claims

Claims 1-10 were filed with the original application, and claims 11-19 were added during prosecution, while the original claims were canceled. Thus, claims 11-19 are pending, stand rejected and are appealed. A copy of the appealed claims is attached as Appendix A.

IV. Status of the Amendments

All proffered amendments have been entered.

V. Summary of the Claimed Subject Matter

Claim 11, the only independent claim, is drawn to a pharmaceutical formulation comprising (a) a nucleic acid, and (b) a nonsteroidal anti-inflammatory drug, wherein said formulation exhibits a pH value from pH 6.2 to pH 7.0, and wherein said nonsteroidal anti-inflammatory drug is present at a concentration within the range from 10 to 500 μmol/l. This claim is supported in the application as filed, for example, at page 8, lines 1 to 19.

VI. Ground of Rejection to be Reviewed on Appeal

Are claims 11-19 obvious under 35 U.S.C. §103 over Chouini-Lalanne *et al.* (Exhibit 1) in view of Ajmone-Cat *et al.* (Exhibit 2) and Gennaro (Exhibit 3)?

VII. Argument

A. Standard of Review

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. §706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312. Accordingly, it necessarily follows that an examiner's position on appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Rejection Under 35 U.S.C. §103

Claims 11-19 are rejected as obvious over Chouini-Lalanne *et al.* in view of Ajmone-Cat *et al.* Chouini-Lalanne is cited as teaching supercoiled phage DNA, 10 mM NaCl, and one of four different NSAIDs. Ajmone-Cat is cited for teaching the popularity of NSAIDs and that flurbiprofen inhibits PGE_2 production. The examiner further argues that it would have been obvious to determine the concentration of chloride ions and the concentration of NSAID in

preparing a pharmaceutical. From this, the examiner concludes all of the pending claims are rendered obvious by these disclosures. Appellants traverse.

Claim 11, the only independent claim, is drawn to "a pharmaceutical formulation comprising (a) a nucleic acid, and (b) a nonsteroidal anti-inflammatory drug, wherein said formulation exhibits a pH value from pH 6.2 to pH 7.0, and wherein said nonsteroidal anti-inflammatory drug is present at a concentration within the range from 10 to 500 μmol/l."

i. The examiner improperly combines the primary and secondary references

The examiner has combined the teachings of Chouini-Lalanne with those of Ajmone-Cat. However, a careful review of these two reference shows that while, the latter certainly provides a listing of NSAIDs and their various therapeutic uses, the former has absolutely nothing to do with therapies, and thus the combination is unworkable when the full scope of both references is relied up.

As has been repeatedly pointed out, Chouini-Lalanne has nothing to do with therapy. The inclusion of an NSAID with a DNA in this reference is for a very specific and limited purpose – to use DNA as a ***target*** for the possible phototoxic actions of NSAIDs. There is nothing in this reference that would lead one to Ajmone-Cat, ***at least for the purpose the examiner relies upon***. Notably, when citing to this secondary reference, the examiner immediately fixates on the therapeutic aspects of the publication, but the question is why? Chouini-Lalanne provides no therapeutic teachings. Thus, if the examiner is to rely on Ajmone-Cat simply for the limited purpose of providing a listing of NSAIDs, which would in theory be permissible, then the rejection is still deficient as providing no guidance regarding therapeutic uses of DNAs combined with NSAIDs, which is the basis for the claimed ***pharmaceutical***

compositions. Alternatively, if the examiner attempts to extract more from Ajmone-Cat, namely its therapeutic aspects, then the combination with Chouini-Lalanne is completely improper, as the latter provides no reason whatsoever to consider therapies.

Thus, the entire basis of the rejection constitutes an improper picking and choosing of those aspects of the references that "work" for the examiner's purposes, and ignoring those that do not. Yet a prior art reference must be considered in its entirety, *i.e.*, as a whole, including portions that would lead away from the claimed invention. *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983). Moreover, if the proposed modification or combination of the prior art would change the principle of operation of the prior art invention being modified, as it does here, then the teachings of the references are not sufficient to render the claims *prima facie* obvious. *In re Ratti*, 270 F.2d 810, 123 USPQ 349 (CCPA 1959). And indeed, where the teachings of two or more prior art references conflict (therapeutic *versus* non-therapeutic), the examiner must weigh the power of each reference to suggest solutions to one of ordinary skill in the art, considering the degree to which one reference might accurately discredit another. *In re Young*, 927 F.2d 588, 18 USPQ2d 1089 (Fed. Cir. 1991).

Thus, the facts of this case, and the case law cited above, clearly illustrate the impropriety of the very combination upon which the entire rejection relies. As such, it is clear that the rejection is improper and should be reversed.

ii. The examiner is improperly ignoring an explicit claim limitation

Moving on, and assuming *arguendo* that the above-noted combination of references can stand, the examiner has indicated that the limitation of pharmaceutical formulation may be properly ignored in interpreting the claims and applying the prior art. Appellants, once again,

submit that this is not true – ***all*** limitations must be considered on their merits. Whether a pharmaceutical formulation would be enough to establish patentability, once considered, is dependent on the facts of a particular case. Indeed, *In re Lerner*, 169 USPQ 51 (CCPA 1971) stands for the proposition that an otherwise unpatentable compound is not rendered patentable by addition of a carrier or diluent. However, if the use of the carrier would not be obvious, the resulting composition ***could*** be patentable. *See In re Riden et al.*, 138 USPQ 112 (CCPA 1963); *Ex parte Billman*, 71 USPQ 253 (POBA 1946); *Ex parte Erdmann et al.*, 194 USPQ 96 (POBA 1975).

In response, the examiner continues to argue that "nothing about the phrase 'pharmaceutical formulation' implies that it can be only for *in vivo* use, and pharmaceutical drugs are routinely tested *in vitro*." This obfuscated argument holds no merit. Appellants are their own lexicographers, and the examiner is ***compelled*** to interpret the claims and terms therein in light of appellants' specification. At page 7, lines 16-24, appellants provide a ***detailed*** explanation of the claim term in question:

> The term "formulation" or "pharmaceutical formulation" as used in the present document means the pharmaceutical form of preparation, for example, for a drug or an inoculation medium, which his administered *in vivo* to a human or an animal, or *in vitro* or *ex vivo* to organs, tissues or cells, consisting of one or more active ingredients and auxilliary formulation agents. Active ingredients according to the present invention are nucleic acids.

There would be little need, given this ***explicit*** definition of "pharmaceutical formulation" to proceed to an extrinsic source, but if that were done, a wholly consistent definition would be found:

> Pharmaceutical formulation, in pharmaceutics, is the process in which different chemical substances are combined to a pure drug substance to produce a final medicinal product.

> Formulation studies involve developing a preparation of the drug which is both stable and acceptable to the patient. For orally taken drugs, this usually involves incorporating the drug into a tablet or a capsule. It is important to appreciate that a tablet contains a variety of other substances apart from the drug itself, and studies have to be carried out to ensure that the drug is compatible with these other substances.

See www.answers.com. Thus, there is ***no question*** that this claim term clearly requires that the claimed subject matter be suitable for administration to a subject *in vivo*. The fact that, having satisfied this more rigorous definition, it may also be used in less rigorous *ex vivo* or *in vitro* contexts, does not ***negate*** the limitation of a higher standard of *in vivo* suitability.

Still, the examiner argues that appellants' definition does not ***exclude*** non-pharmaceutical uses. Again, this comment is completely irrelevant given the definition provided above. Just because a composition of matter may be used in ways that are not intended – in this case for additional purposes not requiring pharmaceutical suitability – does not mean that the examiner is free to ignore explicit limitations that ***are*** part of the claims. This rejection is one for ***obviousness***, not inherent anticipation, and thus the question is ***why*** one would modify the teachings of Chouini-Lalanne, which merely discloses the use of DNA as a ***target*** for possible phototoxic actions of NSAIDs, to prepare a formulation suitable not just for the *in vitro* test that was described by the reference, but for the more rigorous pharmaceutical use of a DNA-NSAID combination as now claimed. As discussed previously, Ajmone-Cat cannot provide any such motivation given that it merely discusses NSAIDs, and thus says nothing about the benefits or requirements of adding DNA to pharmaceutical formulation of an NSAID.

Once again, it is black letter law that the examiner ***must*** take into consideration the pharmaceutical formulation limitation of the present claims, and when properly considered, it is

clear that the cited prior art fails to suggest such an invention. This fact alone establishes the patentability of the rejected claims over the cited art.

iii. The examiner has not established the obviousness of the pH recitation

However, the "pharmaceutical" aspect of the claims is not the only limitation lacking from the art. In addition, claim 1 differs from the teachings of Chouini-Lalanne at least by having a pH range of 6.2-7.0, as opposed to a pH of 7.4. There is no comparable disclosure of pH ranges in Ajmone-Cat, so the question is whether there is sufficient motivation in Chouini-Lalanne alone to modify the pH range from 7.4 to at last 7.0. It is well-established that ***some*** motivation must be provided by the examiner - either in the prior art or in the general knowledge in the field. None is present here.

In order to avoid the need to provide such motivation – which is blatantly lacking from the art – the examiner has argued that the present invention constitutes mere "optimization," which is not patentable. However, appellants submit that even "optimization" requires some motivation, and looking at Chouini-Lalanne, the reference uses a pH 7.4 phosphate buffer to dilute DNA and NSAIDs, and the resulting reaction is performed in a test tube. What motivation is there to drop the pH from the stated 7.4 level to 6.2-7.0? Appellants submit that the answer is ***none***, and hand-waving regarding optimization is insufficient because one can only optimize that for which there is a motivation to cause change. Ajmone-Cat is of no avail as it does not discuss using anything but NSAIDs, and so the question remains – why bother to "optimize" the DNA composition of Chouini-Lalanne when no *in vivo* use for such is contemplated?

In a vain attempt to justify this line of reasoning, the examiner also argued that there is still sufficient motivation from appellants' intended pharmaceutical use to drop the pH from Chouini-Lalanne's 7.4 "to the art accepted physiological neutral pH of 7.0" (emphasis in

original). This approach is doubly objectionable. First, the examiner has denied that there ***is*** any *in vivo* implication for the instant claims. If true, how then would one of skill in the art, looking at appellants' claims, discern *in vivo* intent as the basis for dropping the pH? Thus, entire line of argument is unjustified given the examiner's position above.[1]

Second, appellants have submitted an expert declaration on this very point (Exhibit 3). In that declaration, Dr. Gerhard Burkhardt stated that the adjustment of pH was a critical feature in the delivery of oligonucleotides to cells. Moreover, the stated that "the adjustment of pH in this system is neither trivial nor mere optimization of a routine aspect." Burkhardt Declaration, Para. 7. The examiner's only rebuttal, other than to dismiss the declaration out of hand, is to argue that Gennaro teaches that optimization of pH is an important, if not critical, aspect of drug delivery. This comment is completely irrelevant to the obviousness of ***a specific pH range***! Appellants are not claiming a process of optimizing pH, arguably what Gennaro is talking about, but the discovery of ***an optimized pH for the delivery of ODNs***. Just as with Chouini-Lalanne and Ajmone-Cat, Gennaro says ***nothing*** about this feature of the claims.

Third, and more critically, where is there evidence that physiological pH is 7.0? Indeed, a quick perusal of the internet reveals that physiologic pH is 7.35-7.4 (see www.answers.yahoo.com; en.wiktionary.org), essentially that set forth by Chouini-Lalanne. Hence, again, ***there is no motivation to modify that reference to arrive at the claimed invention***. Appellants have called for an Examiner's Affidavit under 37 C.F.R. §1.104(d)(2) to support reliance on personal knowledge of PTO personnel. No such affidavit has been forthcoming, and thus appellants' factual averments regarding physiologic pH must be admitted.

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[1] Put another way, if the examiner maintains that there is no *in vivo* implication from the term "pharmaceutical," this entire line of argument falls away and the pH limitation alone is enough. Alternatively, if the examiner is compelled to adopt an *in vivo* inference from the term "pharmaceutical," the claim is patentable for the reasons given above, and one need not even reach the question of the pH limitation. Simply put, the examiner cannot have his cake and eat it too.

iv. Unexpected Results

Finally, though not required, appellants again submit that even if there were a *prima facie* case of obviousness, the evidence or record shows a surprising result stemming from lowering of the pH from 7.4 to 7.0, namely, ***an increase in DNA uptake of 50%*** (see FIG. 3). Thus, although no "special property" is required for patentability here, this clearly is one as the examiner has not denied that such an increase would indeed be surprising.

v. Summary

In sum, there is no legal basis for motivation to combine the cited art, even ***general*** motivation, but there are limitations in the claims that would overcome the advanced combiation even if proper. Nor is the presently claimed invention one of optimization, and even if it were, the surprising results of record would still render the claimed invention patentable over the cited art. Therefore, reconsideration and withdrawal of the rejection is therefore respectfully requested.

C. Conclusion

In light of the foregoing, appellants respectfully submit that all pending claims are non-obvious under 35 U.S.C. §103. Therefore, it is respectfully requested that the Board reverse each of the pending rejections.

Respectfully submitted,

Date: May 28, 2009

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VIII. APPENDIX A – APPEALED CLAIMS

1-10. (Canceled)

11. A pharmaceutical formulation comprising (a) a nucleic acid, and (b) a nonsteroidal anti-inflammatory drug, wherein said formulation exhibits a pH value from pH 6.2 to pH 7.0, and wherein said nonsteroidal anti-inflammatory drug is present at a concentration within the range from 10 to 500 µmol/l.

12. The formulation according to claim 11, wherein the pH value is 6.5 or 7.0.

13. The formulation according to claim 11, wherein the formulation has a chloride ion concentration of from 5 to 100 mmol/l.

14. The formulation of claim 13, wherein the chloride ion concentration is from 5 to 10 mmol/l.

15. The formulation of claim 11, wherein the nonsteroidal anti-inflammatory drug is present at a concentration within the range from 50 to 250 µmol/l.

16. The formulation of claim 11, wherein the nonsteroidal anti-inflammatory drug is present at a concentration of 100 µmol/l.

17. The formulation claim 11, wherein the nonsteroidal anti-inflammatory drug is flurbiprofen or indoprofen.

18. The formulation according to claim 1, further comprising a carrier substance or additive.

19. The formulation according to claim 13, further comprising a chloride ion concentration within the range from 5 to 50 mmol/l.

IX. APPENDIX B – EVIDENCE CITED

Exhibit 1 – Chouini-Lalanne *et al.*

Exhibit 2 – Ajmone-Cat *et al.*

Exhibit 3 – Gennaro

Exhibit 4 – Declaration of Dr. Gerhard Burkhardt

X. APPENDIX C – RELATED PROCEEDINGS

None

EXHIBIT 1

Biochemical Pharmacology, Vol. 55, pp. 441–446, 1998.

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Nonsteroidal Antiinflammatory Drug-Photosensitized Formation of Pyrimidine Dimer in DNA

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ABSTRACT. Phototoxic nonsteroidal antiinflammatory drugs (NSAIDs) may induce DNA damage *in vitro* upon irradiation. In this study, we investigated the ability of ketoprofen (KP), tiaprofenic acid (Tia), naproxen (NP) and indomethacin (IND) to photosensitize the formation of pyrimidine dimers and single strand breaks. Both kinds of damage were sought by analyzing DNA-drug mixtures irradiated at 313 nm by agarose gel electrophoresis. The formation of pyrimidine dimers was evidenced by using endonuclease V from bacteriophage T4 and compared to that induced by acetophenone, a well-known photosensitizer of thymine dimerization. Upon irradiation of DNA alone, pyrimidine dimers were observed while single strand breaks were not detected under our conditions. DNA, in the presence of NSAIDs, undergoes single strand breaks, the quantum yield of the DNA cleavage so induced (ΦC) varying from 5×10^{-4} for KP to 10^{-3} for IND. The formation of dimers was only increased in the presence of KP or Tia. The quantum yields of pyrimidine dimers formed by photosensitization (ΦD) were 2×10^{-4} for KP and 10^{-3} for Tia, respectively. The oxygen and concentration dependence of both processes was analyzed in the case of KP. In aerated solution, KP-photoinduced cleavage of DNA was predominant on the photodimerization process of pyrimidines, whereas in deaerated solution the cleavage was decreased and the dimerization increased. These results reflect competition between a radical process leading to DNA cleavage and a poorly efficient energy transfer between the drug and the pyrimidines at the origin of the dimerization process. BIOCHEM PHARMACOL 55;4: 441–446, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. ketoprofen; naproxen; tiaprofenic acid; indomethacin; photocleavage; photodimerization

Among the various drugs which may induce phototoxic effects, the case of nonsteroidal antiinflammatory drugs (NSAIDs)§ is well known. These drugs, widely used in the treatment of arthritic diseases, may promote enhanced cutaneous photosensitivity. After exposure to the sun, patients may develop reactions characterized mainly by erythema, itching, stinging and burning skin. In order to determine the origin of these phenomena, the photosensitization of biological targets by NSAIDs was investigated. It was shown that many NSAIDs which are derived from propionic acid and are the most phototoxic [1, 4], such as benoxaprofen (BNP), naproxen (NP), ketoprofen (KP), tiaprofenic acid (Tia), suprofen and carprofen, induce membrane damage [5, 11]. Photosensitization of DNA damage by NSAIDs has also been established. In particular, Artuso *et al.* have demonstrated that BNP, KP, Tia, NP and diflunisal photoinduce the formation of single strand breaks (SSB) via mainly radical processes [12, 13]. Singlet oxygen, generated by these compounds under irradiation, did not seem to be significantly involved in the formation of SSB. DNA breakage may be facilitated by a noncovalent drug-DNA interaction as recently suggested for suprofen [14] and naproxen (NP) [15].

Until now, only the formation of single strand breaks has been detected during the photosensitization of DNA by NSAIDs except in the case of BNP, where the formation of pyrimidine dimers was also mentioned [13]. From a general point of view, the photosensitization of pyrimidine dimerization by exogenous agents has been poorly investigated even though pyrimidine dimers, which may be at the origin of skin cancer, can be considered as the major lesion resulting from the irradiation of DNA. This led us to undertake an examination of the occurrence of such damage during the photosensitization of DNA by NSAIDs. It was known as early as 1967 that the main photosensitizers of pyrimidine dimer formation are carbonyl compounds such as acetone, acetophenone or benzophenone [16, 20]; therefore, we first focused our study on ketoprofen (KP) and tiaprofenic acid (Tia), which exhibit structures close to that

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§ *Abbreviations:* AC, acetophenone, methyl phenyl methanone; BNP, benoxaprofen, 2-(4-chlorophenyl)-α-methyl-5-benzoxazole acetic acid; IND, indomethacin, 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetic acid; KP, ketoprofen, 2-(3-benzoylphenyl) propionic acid; NP, naproxen, d-2-(6-methoxy-2-naphthyl) propionic acid; NSAIDs, nonsteroidal antiinflammatory drugs; r, drug/DNA base pair molar ratio; S, number of single strand breaks per mole of DNA; SSB, single strand break; Tia, tiaprofenic acid, 2-[4-(2-benzoyl)thiophenyl] propionic acid.

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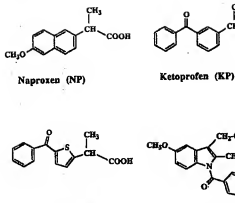

FIG. 1. Structures of the various NSAID compounds tested as DNA photosensitizers.

of benzophenone and are already known to be phototoxic *in vivo* and DNA breaker agents *in vitro*. For comparison, similar studies were also performed on two other NSAIDs: naproxen (NP), a phototoxic propionic acid derivative, and indomethacin (IND), an acetic acid derivative which in contrast reduces UV-induced erythema in normal patients [21, 22] (Fig. 1). Acetophenone (AC), a classical photosensitizer of thymine dimerization, was used as standard. These studies were carried out *in vitro* on ΦX-174 DNA which was irradiated at 313 nm in the presence of the drugs. The formation of cyclobutylpyrimidine dimers was detected using phage T4 endonuclease V, a DNA repair enzyme which selectively cuts DNA next to the pyrimidine dimer sites thus leading to single strand breaks by a two-step mechanism [23].

MATERIALS AND METHODS

Chemicals and Biochemicals

KP was purchased from Specia, Tia from Roussel Uclaf, IND from Merck, NP from Syntex, and AC from Aldrich. Supercoiled ΦX174 DNA (MW 3.6×10^6 Da, 5386 base pairs per molecule) Form I was purchased from Pharmacia. The DNA was used after dilution in 5 mM phosphate buffer (pH 7.4) containing 10 mM NaCl so that the concentration of the solution was 14 nM in DNA molecules or 75.4 μM in bp. The amount of contaminant Form II was checked by agarose gel electrophoresis followed by microdensitometry and was less than 10%. No Form III was detected in the starting material. The electrophoresis-grade agarose was obtained from Touzart and Matignon. DNA concentrations (mg) were determined by UV absorption spectroscopy using a conversion factor of 21 absorbance units/mg of DNA. DNA concentrations in base pairs were determined spectrophotometrically at 260 nm using the extinction coefficient of 13,200 M^{-1} cm^{-1} [24]. Phage T4 endonuclease V was prepared according to the procedure of Lommel *et al.* [23]. Endonuclease activity checked before use was 4.3×10^{12} sites/μL/min. All solutions were prepared in phosphate buffer (5 mM, 10 mM NaCl, pH = 7.4).

Irradiation Procedure

Samples were prepared by mixing 5 μL of 14 nM DNA (75.4 mM bp), 5 μL of drug solution at a fixed concentration in phosphate buffer and 10 μL of phosphate buffer. The sample containing DNA alone was prepared from 5 μL of 14 nM DNA and 15 μL phosphate buffer. The mixture was placed in glass tubes (3 mm diameter) and incubated for 20 min in the dark. For experiments in deaerated conditions, the starting solutions were first bubbled with argon and the tubes containing the mixture then flushed with argon and capped. All the concentrations given in the text are the final concentrations in the tubes. Except for KP experiments using drug concentrations varying from 2.5 to 180 μM, the NSAID concentration was 25 μM. Absorbance at 313 nm in the tube was 8.7×10^{-3} for KP, 9.4×10^{-3} for NP, 50×10^{-3} for IND, 113×10^{-3} for Tia, 0.7 $\times 10^{-3}$ for AC and 1.2×10^{-3} for DNA.

The solutions were irradiated at 313 nm for various periods of time with a Muller reactor device equipped with a 200 W high-pressure mercury lamp (Osram), a water cooling filter and an interference filter (Oriel 313FS 10–50 12% transmission at 313 nm, bandwidth 10 nm). The energy was monitored with an EGG Gamma radiometer-photometer system. The power received by the samples was *ca.* 1.4 10^{-3} W/cm^2.

Photosensitized Cleavage Experiments

After irradiation, 5 μL of a mixture containing 250 mM HEPES pH 7.45, 75% glycerol and 0.05% bromophenol blue were added to the irradiated solution. The sample was then analyzed by electrophoresis on 0.8% agarose horizontal slab gel in Tris borate buffer, and quantification of the various forms of DNA (I, II, III) was performed as described by Arruso *et al.* [12]. The number of single strand breaks per mole of DNA (S) generated by photosensitization was calculated from the relative percentage of Forms I and II, assuming a Poisson distribution and using the formula $S = \ln C/C_o$, where C_o is the initial concentration of DNA in Form I and C the concentration of Form I after irradiation. A coefficient of 1.66 was used to correct the lower efficiency of ethidium bromide binding to DNA to Form I with respect to Form II and III [25]. The quantum yield of photosensitized formation of single strand breaks (ΦC) was calculated from the total number of DNA breaks per sec and the number of photons absorbed during the same time by the drug in the sample. The rate of SSB formation was evaluated using only the linear part of the curve $S = f(t)$ to minimize the possible perturbations induced by the photodegradation of the drug and the occurrence of inefficient cleavage or dimerization reactions on Form II.

Photosensitized Dimerization Experiments

At the end of the irradiation, DNA was precipitated by addition of 100 μL of cold ethanol and 2 μL of 3 M sodium acetate buffer (pH = 5.5) to the irradiated mixture. The samples were kept in dry ice for an hour and centrifuged for 45 min (10,000 × g). The residue was washed in 70% ethanol, centrifuged and dried under vacuum. Twenty μL of diluted phage T4 endonuclease V in Tris-acetate buffer (pH = 8) were added to the residue, and the solution was incubated for 30 min at 37°. Proteins were removed from the mixture by washing with chloroform-phenol-isoamyl alcohol. The samples were further treated by the same procedure as used for the cleavage experiments. Controls used the same procedure without endonuclease and the residue was dissolved in 20 μM phosphate buffer. It could be considered that the number of dimers due to the direct excitation of DNA in the mixture is very likely similar to that obtained in the absence of drug, since the solution was far too dilute to absorb all the light arriving on the sample, both drug and DNA having very low absorbance. In experiments using KP (25 μM) as photosensitizer, only 2.3% of the incident light was absorbed by the solution: 0.27% by DNA and 2% by KP. Consequently, the quantum yield of photosensitized formation of dimers (ΦD) could be evaluated from the number of single strand breaks detected after treatment by phage T4 endonuclease V, after subtraction of the number of single strand breaks obtained before treatment and of those corresponding to dimers resulting from irradiation of DNA alone. The values of quantum yields of both cleavage and dimerization processes obtained by this method were slightly underestimated since these reactions, occurring on Form II and not leading to Form III, were not taken into account.

RESULTS

Comparative Study of the Photosensitization of DNA Damage by Various NSAIDs

NSAID photosensitization of DNA damage was carried out by irradiating at 313 nm phosphate-buffered solutions of KP, Tia, NP, IND and AC (25 μM) containing supercoiled ΦX 174 DNA (Form I, 18.85 μM in base pairs) over periods of up to 40 sec. The formation of SSB was then analyzed by agarose gel electrophoresis. As seen in Fig. 2, all the NSAIDs photosensitized the formation of SSB. Comparison of these effects for the various compounds at the same concentration corresponding to a drug to base pair molar ratio of 1.4 shows that Tia and KP were significantly more efficient than NP, IND or AC stated as reference. In the case of IND and AC, it should be noted that S did not vary with the irradiation time and remained very low. The qualitative data previously reported [12, 13] can thus be quantified. The quantum yields of SSB (ΦC) induced by NSAIDs under irradiation at 313 nm under our conditions varied from 5×10^{-4} for KP to 10^{-5} for IND, while AC exhibited a higher quantum yield (1.4×10^{-3}) (Table 1).

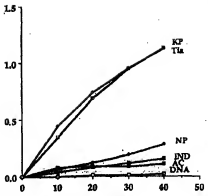

FIG. 2. Influence of irradiation time on the mean number of SSB per DNA molecule (S) photoinduced in the presence of KP, Tia, NP, IND and AC at a same concentration (25 μM) or in the absence of photosensitizer.

Irradiation of DNA alone, under the same conditions, did not induce the formation of SSB, but the action of phage T4 endonuclease V on the solution of irradiated DNA promoted SSB corresponding to the formation of dimers. When DNA was irradiated in the presence of KP or Tia or AC, the number of dimers thus formed was increased. This number was not significantly modified by NP and was slightly reduced by IND. Among the whole set compounds studied, AC was by far the most efficient photosensitizer of pyrimidine dimerization. Regarding only NSAIDs, it appears that only KP and Tia photosensitized the formation of pyrimidine dimers in DNA. The number of dimers due to the photosensitization was plotted versus the irradiation time. Figure 3 shows that the dimers induced by NSAIDs increased slightly with the irradiation time except for NP. In this case, a clear enhancement of the rate of formation of the dimers was observed after a short period of irradiation. This modification of the behavior of NP with the irradiation time may be due to the formation of photodegradation products more efficient than NP itself. The quantum yields of dimerization induced on DNA by NSAID photosensitization ΦD at 313 nm were found to be 2×10^{-4} for KP, 10^{-5} for Tia and 6×10^{-3} for AC for a drug/DNA base pair molar ratio of 1.4. As can be seen in Table 1, NSAIDs mainly photosensitize DNA cleavage. They are less efficient on pyrimidine dimerization.

TABLE 1. Quantum yields of SSB (ΦC) and dimers (ΦD) due to the photosensitization by NSAIDs and AC (25 μM, r = 1.4) at 313 nm

	KP	Tia	NP	IND	AC
$\Phi C \times 10^3$	0.5	0.05	0.1	0.01	1.4
$\Phi D \times 10^3$	0.2	0.01			6

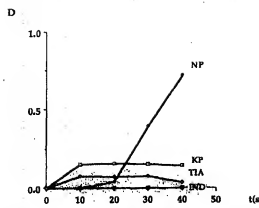

FIG. 3. Influence of irradiation time on the mean number of dimers per DNA molecule (D) photosensitized by KP, Tia, NP and IND.

Influence of the Presence of Oxygen and of Drug Concentration on the Photosensitization of DNA Damage by KP

The influence of various parameters was studied in the particular case of KP. When the same experiments as above were performed under a nitrogen atmosphere, the KP-photosensitized formation of SSB was twofold lower than in the presence of air. In contrast, the number of dimers photosensitized by the drug was increased by a factor of 1.6 (Fig. 4). The presence of oxygen quenched the photosensitized formation of dimers and simultaneously enhanced the formation of SSB.

The influence of the drug concentration on the efficiency of both reactions was studied for a change in KP concentration from 2.5 μM to 185 μM, r varying from 0.14 to 10.3. The number of SSB observed for a constant time (10 sec) of irradiation of DNA (18.85 μM) increased slightly from 0.23 to 0.35 as r was varied from 0.14 to 1.4 and then remained at the same level when r was increased up to 10.3. The photosensitization of pyrimidine dimers by

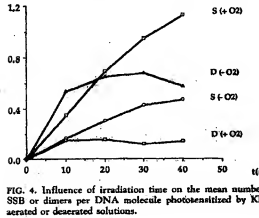

FIG. 4. Influence of irradiation time on the mean number of SSB or dimers per DNA molecule photosensitized by KP in aerated or deaerated solutions.

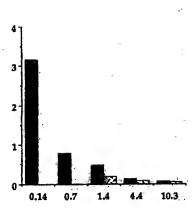

FIG. 5. Influence of the drug to base pair ratio on the quantum yields of formation of SSB (Φ) or dimers (Φ) photosensitized by KP.

KP only occurred when the concentration was sufficiently high. The number of dimers per mole of DNA, induced by the drug for the same time of irradiation, became significant (0.16) only for r = 1.4. Then, it increased to 0.28 when r increased up to 10.3. The quantum yields of both cleavage and dimerization processes decreased as r increased (Fig. 5).

DISCUSSION

Our findings demonstrate that NSAIDs photosensitize DNA damage with different efficiencies depending on the structure of the drug and the type of damage. As previously reported, all the compounds investigated induce DNA cleavage upon irradiation at 313 nm [12–13, 15]. The quantum yields of SSB formation, ΦC, vary from 5×10^{-4} for KP to 10^{-5} for IND at 313 nm for a drug to DNA base pair ratio r = 1.4. They are of the same order of magnitude as the value recently given by Condorelli *et al.* for Suprofen, $\Phi C = 10^{-4}$ for r = 1.8 [14]. The quantum yield determined by De Guidi *et al.* for Naproxen for r < 0.5 was slightly higher, 4.3×10^{-3} [22] than that given here for r = 1.4, which is consistent with the fact that the quantum yield decreases as r increases.

The mechanism proposed for these chain breaks is a Type I mechanism [12–15] involving hydroxyl radicals. It is well known that hydroxyl radicals detected by scavengers are very efficient in chain break production [26]. Such a mechanism has been given by Chatlier *et al.* for benzophenone [16, 27], hydroxyl radicals resulting from hydrogen abstraction by the excited triplet state of benzophenone to a molecule of water. Since then, characterization of the final products of pyrimidine and pyrimidine photosensitization by benzophenone has confirmed the involvement of a Type I mechanism, but indicates that the initial step is more likely an electron transfer from the bases to the triplet-excited benzophenone rather than hydrogen abstraction

[28–30]. Recently, flash-photolysis experiments on benzophenone-adenine mixtures which indicate the formation of the benzophenone radical anion, have supported this hypothesis [31]. Such a mechanism, leading to the formation of a nucleotide radical cation which is rapidly deprotonated to give the neutral radical, could also explain the DNA cleavage observed. The behavior of KP under irradiation, which may be compared to that of benzophenone [32], leads us to propose, as for KP-mediated DNA cleavage, the participation of a charge transfer process. The influence of the presence of oxygen, which increased the yield of chain break production mediated by benzophenone [27], was correlated to a decrease in the rate of benzophenone photolysis in the presence of oxygen. The same effect on ΦC is observed here with KP. Found also by Artuso *et al.* for short irradiation times, it was reversed for longer periods of time.

When DNA is irradiated in the presence of NSAIDs, pyrimidine dimers are formed simultaneously with SSB. However, it appears that only KP and Tia promote the formation of more dimers than those observed by irradiating DNA alone. The quantum yields of dimers arising from photosensitization were *ca.* 2×10^{-4} for KP, 10^{-5} for Tia and 6×10^{-5} for AC. This latter value may be favorably compared to the quantum yield of thymine dimerization photosensitized by acetophenone, 1.6×10^{-3} [33]. In contrast with KP and Tia, NP and IND do not enhance the formation of dimers in DNA upon irradiation, IND leading to a number of dimers lower than that formed in the absence of drug. Thus, it appears that only NSAIDs with a carbonyl function photosensitize the formation of pyrimidine dimers. It is well known that many excited ketones can react with DNA by triplet energy transfer [34, 35]. This requires the triplet state of the photosensitizer to be higher than that of thymine (73 kcal mol^{-1}), which is the mononucleotide lowest in energy [31]. Benzophenone, with a triplet energy of approximately 69 kcal mol^{-1}, is not a good candidate for such a transfer. This explains how benzophenone, as well as benzophenone-like compounds such as KP or Tia, promotes pyrimidine dimers only in a low yield [36]. The competition between electron and energy transfer may also reduce the formation of dimers, since in this case the triplet state is quenched by the electron donor [37]. NP, which may have a triplet state close in energy to that of naphthalene derivatives (59–61 kcal mol^{-1}) [38], cannot sensitize the formation of pyrimidine dimers, as observed. The value of the triplet state energy of IND was not known, but the quenching observed with IND suggests that it would be lower than that of thymine, the energy gap thus allowing energy transfer from an excited state of DNA to the ground state of IND to occur.

The influence of oxygen on the efficiency of photodimerization by KP is in agreement with the involvement of an energy transfer. The decrease in the number of pyrimidine dimers observed in the presence of oxygen may be attributed to quenching of the triplet state of the drug by molecular oxygen. The same effect was observed with dimer photosensitization by benzophenone, acetophenone and acetone [39]. The variation of the drug/DNA base pair molar ratio induces an increase in the number of SSBs or dimers induced after 10 sec of irradiation. In the case of the cleavage reaction, this number reached a constant value for $r > 1.4$ which cannot be attributed to self-quenching, since the concentration of the drug was very low ($\sim 10^{-5}$ M). Another hypothesis may be proposed to explain the presence of this plateau. Recently, De Guidi *et al.* [15] observed a similar pattern of variations for NP-induced DNA cleavage. They assumed that a drug-DNA complex is formed and that only DNA-bound NP molecules are responsible for the cleavage. A similar association may explain the saturation observed in our case.

In conclusion, this paper presents one of the first examples of phototoxic drugs photosensitizing the formation of cyclobutylpyrimidine dimers in DNA *in vitro*. KP and, with a lower efficiency, Tia, simultaneously induce chain breaks and dimers. Their behavior may be considered as resulting from their benzophenone-like structure, which is able to induce both electron and energy transfer to DNA [32, 37]. Their ability to form thymine dimers, which are one of the major lesions of DNA following UV irradiation, make such photosensitizers particularly dangerous.

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EXHIBIT 2

Journal of Neuroscience Research 66:715–722 (2001)

Differential Effects of the Nonsteroidal Antiinflammatory Drug Flurbiprofen and Its Nitric Oxide-Releasing Derivative, Nitroflurbiprofen, on Prostaglandin E_2, Interleukin-1β, and Nitric Oxide Synthesis by Activated Microglia

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Increasing experimental, clinical, and epidemiological studies point to the pivotal role of inflammation in the pathogenesis of acute and chronic neurodegenerative diseases and to the protective effects of nonsteroidal antiinflammatory drug (NSAID) therapies. Nonetheless, NSAID long-term therapies are limited by their significant adverse effects on gastrointestinal tract and kidneys. Nitroflurbiprofen (NO-flurbiprofen) belongs to a novel class of antiinflammatory agents obtained by derivatization of conventional NSAIDs with a nitric oxide (NO)-releasing moiety, which strongly reduces their untoward side effects without altering the antiinflammatory effectiveness. The recent evidence of neuroprotective effects of NO-NSAIDs in animal models of chronic brain inflammation prompted us to investigate the activities of NO-flurbiprofen and its parent molecule flurbiprofen on activated rat microglia, the brain resident macrophages. We found that NO-flurbiprofen was as potent as flurbiprofen in preventing prostaglandin E_2 synthesis in lipopolysaccharide-activated microglial cultures. At variance with previous observations on peripheral macrophages is that NO-flurbiprofen did not show any additional capacity to inhibit interleukin-1β synthesis compared with flurbiprofen. Moreover, NO enhanced the expression of the inducible NO synthase; this effect was most likely attributable to the NO released from the drug, as suggested by experiments performed in the presence of the NO donor Deta-NONOate, which similarly to NO-flurbiprofen is characterised by a slow and long-lasting release. Our findings indicate that NO-NSAIDs may differently affect peripheral and brain macrophages. Given their potential therapeutic role in brain inflammation, further in vivo and in vitro studies are required to understand fully their mechanism of action in the CNS. J. Neurosci. Res. 66: 715–722, 2001. © 2001 Wiley-Liss, Inc.

Key words: cyclooxygenase-2; inducible nitric oxide synthase; brain macrophages

Nonsteroidal antiinflammatory drugs (NSAIDs) are among the most widely used therapeutic agents for the treatment of pain, fever, and inflammation. Their beneficial effects are largely attributed to the suppression of prostaglandin (PG) synthesis at sites of inflammation, through the inhibition of the activity of the PG biosynthetic enzyme, cyclooxygenase (COX; Smith and Willis, 1971; Vane, 1971).

On the other side, long-term therapy with NSAIDs is associated with significant adverse effects on the gastrointestinal tract and the kidneys that strongly limit their clinical use (Soll et al., 1991; Segasothy et al., 1994). Among the several strategies adopted to reduce these untoward effects, a promising approach is based on the addition of a nitric oxide (NO)-releasing moiety, through an ester linkage, to conventional NSAIDs (e.g., aspirin, diclofenac, flurbiprofen). The rationale behind this derivatization is the exploitation of the vasoactive property of NO and of its ability to inhibit leukocyte adherence to the mucosal vascular endothelium and protect the gastric mucosa against damage induced by irritant agents (Mac Naughton et al., 1989; Kitagawa et al., 1990). In vivo and in vitro studies (Wallace et al., 1994a,b; Cirino et al., 1996; Fiorucci et al., 1999a) have shown that NO-releasing NSAIDs show a reduced gastrointestinal toxicity profile while retaining intact, or even enhanced, antiinflammatory effectiveness of the parent compounds. As an example, nitroflurbiprofen (NO-flurbiprofen), the nitroderivative of flurbiprofen, suppresses systemic PG synthesis and COX-1 and COX-2 activities as effectively as the parent

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NSAID (Mitchell et al., 1994; Santini et al., 1996) and, in addition, displays an in vivo- and in vitro-enhanced antithrombotic activity (Wallace et al., 1994a; Cirino et al., 1995).

In recent years, the important role of inflammation and glial activation in the pathogenesis of several brain disorders has been clearly established, and epidemiological and experimental data have suggested a beneficial role of NSAIDs in both chronic and acute neuropathologies (McGeer and McGeer, 1999). To date, little is known concerning the effect of NO-releasing NSAIDs on brain inflammation. Recent in vivo studies, based on a model of chronic brain inflammation elicited by infusion of bacterial endotoxin (lipopolysaccharide; LPS) within the CNS of young rats, indicate that daily peripheral administration of NO-flurbiprofen reduces the number of activated microglial cells as judged by the decreased expression of major histocompatibility complex (MHC) II antigens (Hauss-Wegrzyniak et al., 1998) and protects cholinergic neurons of the basal forebrain (Wenk et al., 2000).

In the present study, we compared the effects of NO-flurbiprofen and flurbiprofen on purified rat microglial cultures activated by exposure to a proinflammatory agent such as LPS to examine whether these drugs could differentially modify some important functions associated with microglial activation, such as PG, interleukin-1β (IL-1β), and NO generation (Chao et al., 1992; Minghetti and Levi, 1995). We found that NO-flurbiprofen and flurbiprofen were able to inhibit PGE_2 production to a similar extent. Furthermore, NO-flurbiprofen compared with the parent molecule did not show any additional ability to inhibit IL-1β synthesis, but rather it enhanced the LPS-dependent inducible NO synthase (iNOS) expression.

MATERIALS AND METHODS

Reagents

All cell culture reagents were from Gibco (Grand Island, NY), except for RPMI 1640 medium, which was from HyClone (Cramlington, United Kingdom), and Glutamax I supplement containing L-alanyl-glutamine, which was from Life Technologies (San Giuliano Milanese, Italy). Flurbiprofen and HCT1026 (NO-flurbiprofen) were kindly provided by NicOx (Paris, France) and dissolved in dimethylsulfoxide. The BCA protein assay was from Pierce (Rockford, IL). The ELISA kit for rat IL-1β was from Endogen Inc. (Woburn, MA). Purified COX-2 (from sheep placenta) and iNOS (from mouse macrophages) and specific antibodies for COX-2 and iNOS were obtained from Cayman Chemical Company (Ann Arbor, MI). The [^{3}H]PGE_2 (specific activity 164 Ci/mmol) and western blot enhanced chemiluminescence (ECL) detection system were from Amersham International (Amersham, United Kingdom). Deta-NONOate was obtained from Alexis (Läufelfingen, Switzerland). The monoclonal antibody MRC OX-42 was purchased from Serotec (Oxford, United Kingdom). Specific antibody for PGE_2 and all other chemicals, including LPS (from *Escherichia coli*, serotype 026:B6), were from Sigma (St. Louis, MO).

Cell Cultures

Microglial secondary cultures were prepared from 10–14 day mixed primary glial cultures obtained from the cerebral cortex of 1-day-old rats, as previously described (Levi et al., 1993) and in accordance with European Communities Council Directive No. 86/609/EEC. Microglial cells, harvested from the mixed primary glial cultures by mild shaking, were resuspended in basal Eagle's medium (BME) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, and 100 μg/ml gentamicin and plated on uncoated plastic wells at a density of 1.25×10^5 cells/cm^2. Cells were allowed to adhere for 20 min and then washed to remove nonadhering cells. After 24 hr of incubation, the medium was replaced with fresh medium containing the substance(s) under study. The cultures consisted of ≥99% microglia/macrophages (positive for the macrophage marker ED1) and cell viability was greater than 95%, as tested by trypan blue exclusion.

The mouse monocytic/macrophagic cell line RAW 264.7 was maintened in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM Glutamax I and seeded on uncoated plastic wells at a density of 6×10^5 cells/cm^2. After 24 hr of incubation, the medium was replaced with fresh medium containing the substance(s) under study.

PGE_2, IL-1β, and NO Determination

The supernatants of cells incubated for different intervals with or without LPS and/or other agents were collected, centrifuged, and stored at −20°C until tested. PGE_2 content was quantified using a specific radioimmunoassay (Minghetti and Levi, 1995). The level of PGE_2 present in 10% FCS-containing media was less than 25 pg/ml (assay detection limit). The levels of IL-1β were assayed by specific ELISA, following the manufacturer's instructions. The range of determination was 10–1,000 pg/ml.

The production of NO was determined by measuring the content of nitrite, one of the end products of NO oxidation, by a procedure, modified by Tracey (1992), based on the diazotidation of nitrite by sulphanilic acid (Griess reaction) as previously described (Minghetti et al., 1996). Briefly, 40 μl of 5 mM sulphanilamide, 10 μl of 2 M HCl, and 20 μl of 40 mM N-(1-naphthyl)-ethylene-diamine were added to 150 μl of culture medium. After 10 min of incubation in the dark, the absorbance at 490 nm was measured by a microplate spectrophotometer. A standard nitrite curve (0–50 μM) was generated in the same fashion using a 10 mM solution of $NaNO_2$. The detection limit was 0.25 μM.

Western Blot Analysis

Cell lysates were prepared as previously described (Minghetti and Levi, 1995), and protein concentration was measured by BCA protein assay. Equal amounts of proteins (25 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 10% nonfat milk and incubated with monoclonal anti-COX-2 (1:500) and anti-iNOS (1:2,500) antibodies for 1 hr at 25°C. Horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000; 1 hr at 25°C) and ECL reagents were used as a detection system. Purified COX-2 and iNOS were used as standard controls (0.5 μg/lane).

The optical density of the bands (integrated area, arbitrary units) was measured with a GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA) and referred to the corresponding control samples (taken as 100%), which were run in the same gel.

Immunofluorescence

Microglial cells were plated on coverslips at 1.25×10^5 cells/cm^2 density and incubated with or without LPS and/or 1 μM flurbiprofen or NO-flurbiprofen for 24 hr. At the end of the treatment, the cells were stained with primary monoclonal antibody OX-42 [1:50; 40 min at room temperature (RT)], which recognizes the type 3 complement receptor (CR3), then fixed in 4% paraformaldehyde. Binding of primary antibodies was then revealed by fluorescein-conjugated goat anti-mouse IgG [F(ab')2 fragment]. Coverslips were mounted in DABCO [1,4-diazabicyclo(2.2.2.octane)] solution and examined using a Polyvar fluorescence ultramicroscope (Reichert).

Statistical Analysis

Data are expressed as mean ± SEM, with the number of independent experiments (run in duplicate) indicated in parenthesis, i.e., (n). Comparison between treatment groups was made by paired Student's *t*-test. A two-tailed probability of less than 5% (i.e., $P < 0.05$) was taken as statistically significant.

RESULTS

To study the effects of NO-flurbiprofen and flurbiprofen on microglial functions, cells were incubated for 24 hr with or without 10 ng/ml LPS in the presence of increasing amounts of the two drugs (0.01–10 μM). As shown in Figure 1A, unstimulated microglial cells presented a rounded and simple morphology, as typically described for purified cultures (Visentin et al., 1995). LPS treatment caused only a modest morphological alteration; cell bodies were less spread out and had few thick processes (Fig. 1B). The two drugs, at 1 μM concentration, did not affect cell morphology under unstimulated conditions (Fig. 1C,E), whereas they partly reverted LPS-induced morphology (Fig. 1D,F).

In the concentration range tested (0.01–10 μM), neither drug affected PGE_2 basal level (not shown), but both prevented the LPS-induced PGE_2 accumulation in a dose-dependent manner (Fig. 2). At a 0.1 μM dose, flurbiprofen effectively inhibited the LPS-induced PGE_2 (46% ± 4% of inhibition, mean ± SEM, n = 4), whereas NO-flurbiprofen did not show significant inhibition (11% ± 5%, n = 4). At a 1 μM dose, the two compounds were equally able to down-regulate the LPS-induced PGE_2 synthesis (62% ± 6% and 63% ± 9% of inhibition for flurbiprofen and NO-flurbiprofen, respectively, n = 5). Higher concentrations (5 and 10 μM) reduced PGE_2 production by more than 80% but only moderately affected cell viability; therefore, the 1 μM dose was adopted for the following investigations. The extent of inhibition of COX activity remained essentially unchanged when the analysis was extended for up to 48 and 72 hr of treatment, and no difference between NO-flurbiprofen and its native compound was detectable (not shown; n = 3). In addition, western blot analysis (Fig. 3) showed that the drugs (1 μM) did not affect the expression of COX-2, the inducible isoform of PG's biosynthetic enzyme (95% ± 13% and 114% ± 24% of LPS-induced COX-2 expression for NO-flurbiprofen and flurbiprofen, respectively, n = 5), which has been shown to be the major isoform in activated microglia (Minghetti and Levi, 1995).

We then evaluated the effects of the two NSAIDs on a further important product secreted by activated microglia, the proinflammatory cytokine IL-1β. When microglial cells were stimulated for 24 hr with 10 ng/ml LPS, the IL-1β content in the culture supernatants was 510 ± 167 pg/ml (n = 5), whereas it was undetectable in unstimulated cultures. The level of IL-1β in the supernatants from LPS-stimulated microglial cultures was moderately but significantly depressed by both flurbiprofen and NO-flurbiprofen (1 μM). The inhibitory activity of the two drugs was comparable, being 29% ± 13% and 20% ± 4% for flurbiprofen and NO-flurbiprofen, respectively ($P < 0.05$ and $P < 0.005$ vs. LPS for flurbiprofen and NO-flurbiprofen, respectively, n = 5), suggesting that low and long-lasting levels of NO do not regulate IL-1β production in microglial cells. To support this hypothesis further, we investigated the regulation of microglial IL-1β synthesis by Deta-NONOate, an NO donor characterized by slow release kinetics (half-life 20 hr at 37°C, pH 7.4; Keefer et al., 1996) similar to that of NO-flurbiprofen (Santini et al., 1996). We found that 1 μM Deta-NONOate did not affect the LPS-induced IL-1β secretion and did not further enhance the inhibitory effect of flurbiprofen, consistently with the lack of additional activity of NO-flurbiprofen compared with flurbiprofen.

Finally, we investigated the possible modulatory effect of flurbiprofen and NO-flurbiprofen on NO synthesis, another function related to microglial activation (for review see Minghetti and Levi, 1998). As shown in Table 1, after 24 hr in the presence of LPS, NO synthesis was not affected by the two drugs (1 μM). However, after 48 and 72 hr of stimulation, flurbiprofen, but not NO-flurbiprofen, caused a consistent decrease of nitrite accumulation. The basal level of NO production was unaffected by the two drugs (not shown). To unmask the possible contribution of NO released by NO-flurbiprofen to the nitrite levels, we performed parallel experiments in the presence of the NO-synthase inhibitor NMMA (200 μM), to abrogate the endogenous NO production induced by LPS (Minghetti et al., 1996). In the presence of LPS and NMMA, NO-flurbiprofen was responsible for a small increase in nitrite accumulation (0.37 ± 0.18 μM, n = 5), which could not account for the differences in the levels of nitrite reported in Table 1, in the presence of NO-flurbiprofen or flurbiprofen.

To investigate further the differential effects of NO-flurbiprofen and its parent molecule on the NO synthetic pathway, we analyzed by western blotting the expression of iNOS, the inducible enzyme synthesising NO in LPS-activated microglia (Fig. 4). Interestingly, we found that LPS-induced iNOS expression was increased by NO-flurbiprofen (142% ± 19%, n = 5; $P < 0.05$ vs. LPS and

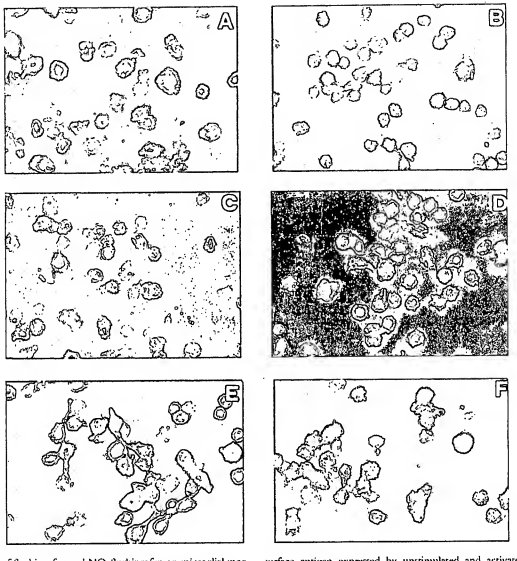

Fig. 1. Effect of flurbiprofen and NO-flurbiprofen on microglial morphology. Microglial cells were treated for 24 hr with or without 1 μM flurbiprofen (F) or NO-flurbiprofen (NO-F) in the presence or in the absence of 10 ng/ml LPS. Cell morphology was analyzed by immunofluorescence staining for the type 3 complement receptor (CR3), a surface antigen expressed by unstimulated and activated microglia. **A:** Microglial cells in nonstimulated conditions; **B:** 10 ng/ml LPS-stimulated cells; **C,E:** nonstimulated cells incubated with 1 μM NO-F or F; **D,F:** LPS-stimulated cells incubated with 1 μM NO-F or F.

$P < 0.0025$ vs. flurbiprofen), whereas flurbiprofen was devoid of effect (108% ± 20%, n = 5; Fig. 4A). The NO-donor Deta-NONOate (1 μM), alone or with 1 μM flurbiprofen, caused an enhancement of LPS-induced iNOS expression (Fig. 4B) comparable to that elicited by 1 μM NO-flurbiprofen (139% ± 11% and 134% ± 8% for Deta-NONOate and Deta-NONOate plus flurbiprofen, respectively, n = 3; $P < 0.05$ vs. LPS-induced iNOS), suggesting that NO released from either NO-flurbiprofen or Deta-NONOate, by increasing iNOS expression, could counteract the inhibitory effect of the flurbiprofen moiety on iNOS enzymatic activity (Table I, Fig. 4).

To verify whether the effect of NO-flurbiprofen on microglial NO production could be particular to this brain macrophage population, we compared microglia with the murine cell line RAW 264.7, a widely used model for peripheral macrophages. RAW 264.7 cells were stimulated with 1 μg/ml LPS, known to evoke PGE_2 and NO production (Guastadisegni et al., 1997), in the presence or in the absence of increasing doses (1–250 μM) of NO-flurbiprofen. The NO-NSAID efficiently abolished the LPS-induced PGE_2 synthesis, even at the lowest concentration used (94% ± 1% of inhibition at 1 μM concentration, n = 6). At variance with the case for microglia

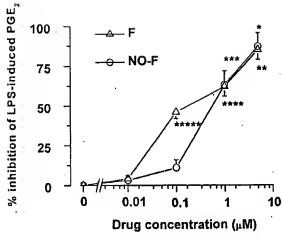

Fig. 2. Dose-response curves for inhibition of LPS-induced PGE_2 production in rat microglial cells by either flurbiprofen or NO-flurbiprofen. Supernatants from microglial cultures exposed to increasing doses of either flurbiprofen (F) or NO-flurbiprofen (NO-F) in the presence or in the absence (not shown) of 10 ng/ml LPS were collected after 24 hr of treatment. PGE_2 content was detected by specific radioimmunoassay. Data reported are means ± SEM from five independent experiments run in duplicate. LPS-induced PGE_2 levels were 330 ± 120 pg/ml ($n = 5$). $^*P < 0.05$ and $^{***}P < 0.005$ for NO-F plus LPS vs. LPS; $^{**}P < 0.025$, $^{****}P < 0.0025$, and $^{*****}P < 0.001$ for F plus LPS vs. LPS.

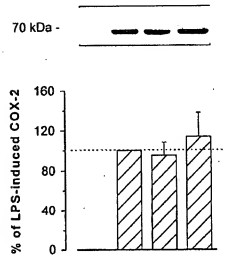

Fig. 3. Effect of flurbiprofen or NO-flurbiprofen on LPS-stimulated COX-2. Microglial cells were treated for 24 hr with or without 1 μM flurbiprofen (F) or NO-flurbiprofen (NO-F) in the presence or in the absence of 10 ng/ml LPS. Cell lysates were prepared, and equal amounts of proteins were analyzed by western blot using anti-COX-2 antibodies. Protein bands were visualized using horseradish peroxidase-conjugated secondary antibodies and ECL. **Top:** One experiment representative of five is shown. **Bottom:** The intensity of the bands was measured by densitometry and the value (arbitrary units) for LPS-induced COX-2 taken as 100%. Means ± SEM of the percentages obtained in five independent experiments are shown.

(Fig. 5A), it reduced the LPS-induced NO production in a dose-dependent manner (Fig. 5B). The percentages of inhibition of LPS-induced nitrite accumulation were 26% ± 6%, 45% ± 8%, and 73% ± 2% for 1, 50, and 250 μM NO-flurbiprofen, respectively ($n = 6$). RAW 264.7 cellular viability was not affected by any of the concentrations tested.

DISCUSSION

NO is an extremely reactive molecule that can directly or indirectly interact with a variety of targets, including transcriptional regulators, enzymes, and receptors (Stamler, 1994; Melino et al., 1997; Dimmeler et al., 1997; Dimmeler and Zeiher, 1997; Clementi et al., 1998; Fiorucci et al., 1999b). The derivatization of NSAIDs with an NO-releasing moiety may widen the pharmacological potentials of these drugs, limiting the untoward side effects of the native compounds and even improving their beneficial antiinflammatory, analgesic, or antipyretic activities, as shown in several studies using models of peripheral inflammation.

To gain information on the potential therapeutic exploitation of NO-NSAIDs in brain inflammation, we have analyzed and compared the effects of the NSAID flurbiprofen and its derivative compound NO-flurbiprofen on some functions of activated microglial cells, such as PGE_2, IL-1β, and NO biosynthesis.

We found that, at micromolar concentrations, flurbiprofen and NO-flurbiprofen were equally potent for inhibiting LPS-stimulated PGE_2 production, without altering the expression of the induced COX-2. These observations are consistent with previous studies on human monocytes performed at comparable drug concentrations (Santini et al., 1996) and confirm that NO-flurbiprofen retains its full ability to inhibit PGE_2 synthesis by preventing COX activity rather than COX expression.

At doses abrogating PGE_2 accumulation by more than 60%, both flurbiprofen and NO-flurbiprofen were able to inhibit, though moderately, the LPS-induced IL-1β release. In apparent conflict with this observation, it has been recently demonstrated that another NO-NSAID (i.e., NO-aspirin), but not its parent molecule, decreased IL-1β secretion in mouse and human LPS-challenged macrophages (Fiorucci et al., 1999b, 2000). This inhibitory effect was attributed to the S-nitrosylation and consequent inhibition of ICE/caspase 1 activity required to process the IL-1β precursor into its active secreted form. The inhibition of caspase activity was also shown for other NO-NSAIDs, including NO-flurbiprofen. In keeping with this view are several observations made with LPS-

TABLE 1. Effect of Flurbiprofen and NO-Flurbiprofen on LPS-Induced Nitric Oxide Production†

	Nitrite (% of LPS; means ± SEM)		
	24 Hours	48 Hours	72 Hours
F (1 μM)	83 ± 31	84 ± 2**	63 ± 10*
NO-F (1 μM)	88 ± 18	97 ± 5	96 ± 9

†Microglial cells (1.25×10^5 cells/well) were subcultured for 24 hr in 10% FCS-containing medium, which was replaced with 400 μl of fresh medium before stimulation. Cultures were then incubated with 1 μM flurbiprofen (F) or NO-flurbiprofen (NO-F) in the presence of 10 ng/ml LPS. Supernatants were collected after 24, 48, and 72 hr and analyzed for nitrite accumulation. Data, expressed as percentages of LPS-induced nitrite accumulation, are means ± SEM from three independent experiments run in duplicate. In the presence of LPS alone, nitrite accumulation was 2.52 ± 0.66 μM, 4.36 ± 1.60 μM, and 12.61 ± 5.43 μM at 24, 48, and 72 hr, respectively (in the absence of LPS, with or without F or NO-F, nitrite levels were undetectable).

*$P < 0.05$ vs. LPS and vs. LPS plus NO-F.

**$P < 0.01$ and $P < 0.05$ vs. LPS or vs. LPS plus NO-F, respectively.

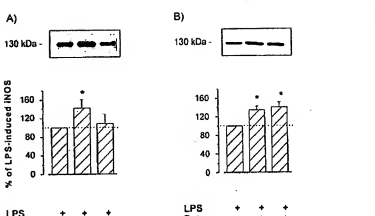

Fig. 4. Effect of flurbiprofen, NO-flurbiprofen, or Deta-NONOate on LPS-stimulated iNOS expression. **A:** Microglial cells were treated for 24 hr with or without 1 μM flurbiprofen (F) or NO-flurbiprofen (NO-F) in the presence or in the absence of 10 ng/ml LPS. Cell lysates were prepared and equal amounts of proteins analyzed by western blot, as for Figure 2. **Top:** One experiment representative of five. **Bottom:** The intensity of the bands was measured by densitometry and the values (arbitrary units) for LPS-induced iNOS taken as 100%. Means ± SEM of the percentages obtained in five independent experiments are shown. *$P < 0.05$ and $P < 0.0025$ vs. LPS or vs. LPS plus F, respectively. **B:** Microglial cells were treated for 24 hr with 1 μM Deta-NONOate with or without 1 μM flurbiprofen (F) in the presence or in the absence (not shown) of 10 ng/ml LPS. Cell lysates were prepared and proteins analyzed by western blot as described above. **Top:** One experiment representative of three is shown. **Bottom:** The intensity of the bands (means ± SEM from three independent experiments, calculated as described above) is shown. *$P < 0.05$ vs. LPS induced iNOS.

stimulated peripheral macrophages indicating that the synthesis of IL-1α and -1β can be inhibited by NO in an autocrine or paracrine fashion (Obermeier et al., 1999). The different behavior of microglial cells could be due to the atypical phenotype of these brain resident macrophages, as demonstrated under other circumstances (Guastadisegni et al., 1997; Minghetti et al., 1999). Moreover, the observation that Deta-NONOate, alone or with flurbiprofen, did not have any effect on IL-1β secretion in LPS-stimulated microglial cells further supports the notion that, in this cell type, IL-1β is not regulated by low and long-lasting NO levels.

At variance with the effects on the prostanoid cascade and IL-1β production, flurbiprofen and NO-flurbiprofen showed distinct effects on LPS-induced NO generation and iNOS expression. Indeed, flurbiprofen, but not NO-flurbiprofen, significantly reduced the LPS-induced nitrite accumulation after 48 and 72 hr of treatment. On the opposite, the LPS-induced iNOS expression was unaffected by flurbiprofen but significantly increased by NO-flurbiprofen. This unique effect of NO-flurbiprofen was most likely due to the NO released by the drug, as suggested by the similar enhancing activity of Deta-NONOate when added to LPS-activated microglia, alone or in combination with flurbiprofen. Therefore, we suggest that the NO released from the NO moiety, by enhancing the expression of iNOS, could counteract the inhibitory effect of the native molecule flurbiprofen on iNOS enzymatic activity. The mechanism by which the NO released by NO-flurbiprofen enhances iNOS expression in microglial cells is still unknown. We have previously shown that iNOS expression is slightly decreased by the NO donor SIN-1 (Minghetti and Levi, 1998; and unpublished observations). However, it is known that concentration, release kinetics, and concomitant presence of superoxide are critical factors in determining the biological effects of NO (Stamler, 1994). As for IL-1β production, NO-flurbiprofen showed an effect on the microglial NO-generating system different from that reported for other cellular populations.

Indeed, NO-flurbiprofen has been shown to inhibit iNOS expression or activity in the murine macrophage cell line J774.2 (Cirino et al., 1996) and in rat neutrophils in vivo (Mariotto et al., 1995). In both cases, the inhibitory activity was ascribed to the NO released by NO-flurbiprofen, which could probably act by preventing the activation of the transcription factor nuclear factor (NF)-κB, involved in the trascriptional control of iNOS (Colasanti et al., 1995; Peng et al., 1995). As for J774.2, we found, in contrast to microglia, that NO-flurbiprofen caused a dose-dependent inhibition of LPS-induced NO synthesis in RAW 264.7 cells, a widely used model for peripheral macrophages. Once more microglial cells, in spite of their close relationship to peripheral macrophages, may respond in a peculiar way to analogous treatment, as a result of their adaptation to the unique cerebral environment (Minghetti and Levi, 1998).

In conclusion, our data indicate that the addition of an NO moiety to the NSAID flurbiprofen does not impair the ability of the drug to inhibit some functions of acti-

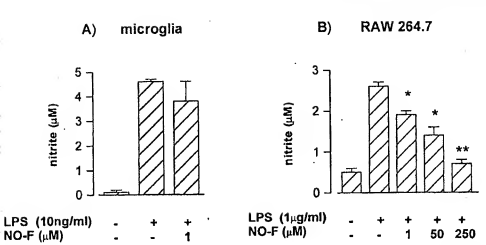

Fig. 5. Effect of NO-flurbiprofen on nitrite accumulation in LPS-activated microglial and RAW 264.7 cultures. Microglial cells (A) and RAW 264.7 cells (B) were subcultured for 24 hr in 10% FCS-containing medium, which was replaced with 400 μl of fresh medium before stimulation. Cultures were then incubated with or without NO-flurbiprofen (NO-F) in the presence of LPS (10 ng/ml or 1 μg/ml for microglia and for RAW, respectively). Supernatants were collected after 24 hr and analyzed for nitrite accumulation. Data reported are means ± SEM from six independent experiments. $*P < 0.05$ and $**P < 0.005$ vs. LPS-induced nitrite accumulation.

vated microglia, such as the production of PGs and IL-1β, which play an important role in brain inflammation. More intriguing is the ability of NO-flurbiprofen, but not of flurbiprofen, to increase the expression of iNOS in LPS-activated microglia. Whether this effect also occurs in vivo remains to be determined. NO is a well-recognized mediator of tissue damage in inflammatory and autoimmune diseases, but, recently, immunomodulatory functions have also been ascribed to NO. As was previously mentioned, its biological effects depend on its local concentration, the cellular microenvironment, and the stage and type of disease (for review see Kolb and Kolb-Bachofen, 1998). Therefore, it is difficult to speculate on the possible final outcome of NO-flurbiprofen enhancement of microglial iNOS expression. Given the robust evidence for a reduced gastrotoxicity profile of NO-flurbiprofen, further in vitro and in vivo studies are required to elucidate the potential therapeutic role of the NO-NSAID in brain inflammation.

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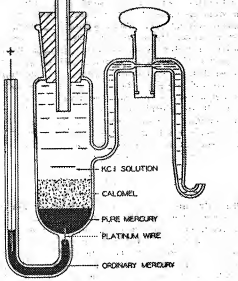

Fig 4. Calomel electrode.

when the normal hydrogen electrode chain shown in Fig 3 is used, it is necessary to subtract the potential due to the calomel electrode itself from the observed voltage. As the magnitude of this voltage depends on the concentration of potassium chloride in the calomel-electrode electrolyte, it is necessary to know the concentration of the former. For most purposes a saturated potassium chloride solution is used which produces potential difference of 0.2488 V. Accordingly, before using Eq 85 for the calculation of pH from the voltage of a cell made up of a calomel and a hydrogen electrode dipping into the solution to be tested, 0.2488 V must be subtracted from the observed potential difference. Expressed mathematically, Eq 91 is used for calculating pH from the potential difference of such a cell.

$$\mathrm{pH} = \frac{E - 0.2488}{0.0591} \qquad (91)$$

In measuring the potential difference between the electrodes, it is imperative that very little current be drawn from the cell, for with current flowing the voltage changes, owing to polarization effects at the electrode. Because of this it is not possible to make accurate measurements with a voltmeter which requires appreciable current to operate it. In its place a potentiometer is used which does not draw a current from the cell being measured.

There are many limitations to the use of the hydrogen electrode:

It cannot be used in solutions containing strong oxidants such as ferric salts, dichromates, nitric acid, peroxide or chlorine or reductants, such as sulfurous acid and hydrogen sulfide.

It is affected by the presence of organic compounds which are reduced fairly easily.

It cannot be used successfully in solutions containing cations that fall below hydrogen in the electrochemical series.

Erratic results are obtained in the measurement of unbuffered solutions unless special precautions are taken.

It is troublesome to prepare and maintain.

Since other electrodes more convenient to use now are available, the hydrogen electrode today is used rarely. Nevertheless, it is the ultimate standard for pH measurements.

To avoid some of the difficulties with the hydrogen electrode, the *quinhydrone* electrode was introduced and was popular for a long time, particularly for measurements of acid solutions. The unusual feature of this electrode is that it consists of a piece of gold or platinum wire or foil dipping into the solution to be tested, in which has been dissolved a small quantity of quinhydrone. A calomel electrode may be used for reference, just as in determinations with the hydrogen electrode.

Quinhydrone consists of an equimolecular mixture of quinone and hydroquinone; the relationship between these substances and hydrogen-ion concentration is

$$\text{Quinone} + 2\ \text{hydrogen ions} + 2\ \text{electrons} \rightleftharpoons \text{hydroquinone}$$

In a solution containing hydrogen ions the potential of the quinhydrone electrode is related logarithmically to hydronium-ion concentration if the ratio of the hydroquinone concentration to that of quinone is constant and practically equal to one. This ratio is maintained in an acid solution containing an excess of quinhydrone, and measurements may be made quickly and accurately; however, quinhydrone cannot be used in solutions more alkaline than pH 8.

An electrode which, because of its simplicity of operation and freedom from contamination or change of the solution being tested, has replaced both the hydrogen and quinhydrone electrodes is the *glass electrode*. It functions by virtue of the fact that when a thin membrane of a special composition of glass separates two solutions of different pH, there is developed across the membrane a potential difference which depends on the pH of both solutions. If the pH of one of the solutions is known, the other may be calculated from the potential difference. In practice, the glass electrode usually consists of a bulb of the special glass fused to the end of a tube of ordinary glass. Inside the bulb is placed a solution of known pH, in contact with an internal silver–silver chloride or other electrode. This glass electrode and another reference electrode are immersed in the solution to be tested and the potential difference is measured. A potentiometer providing electronic amplification of the small current produced is employed. The modern instruments available permit reading the pH directly and provide also for compensation of variations due to temperature in the range of 0–50° and to the small but variable asymmetry potential inherent in the glass electrode.

Pharmaceutical Significance

In the broad realm of knowledge concerning the preparation and action of drugs few, if any, variables are so important as pH. For the purpose of this presentation, four principal types of pH-dependence of drug systems will be discussed: solubility, stability, activity and absorption.

Drug Solubility

If a salt, NaA, is added to water to give a concentration C_s, the following reactions occur:

$$\mathrm{Na^+A^-} \xrightarrow{H_2O} \mathrm{Na^+ + A^-}$$

$$\mathrm{A^- + H_2O \rightleftharpoons HA + OH^-}$$

If the pH of the solution is lowered, more of the A^- would be converted to the unionized acid, HA, in accordance with Le Chatelier's principle. Eventually, a pH will be obtained, below which the amount of HA formed exceeds its aqueous solubility, S_0, and the acid will precipitate from solution; this pH can be designated as pH_p. At this point, at which the amount of HA formed just equals S_0, a mass balance on the total amount of drug in solution yields

$$C_s = [\mathrm{HA}]_p + [\mathrm{A^-}] = S_0 + [\mathrm{A^-}] \qquad (92)$$

Replacing $[A^-]$ as a function of hydronium-ion concentration gives

$$C_s = S_0 + \frac{K_a C_s}{[\mathrm{H_3O^+}]_p + K_a} \qquad (93)$$

where K_a is the ionization constant for the conjugate acid, HA, and $[H_3O^+]_p$ refers to the hydronium-ion concentration above which precipitation will occur. This equation can be rearranged to give

$$[H_3O^+]_p = K_a \frac{S_0}{C_x - S_0} \tag{94}$$

Taking logarithms gives

$$pH_p = pK_a + \log \frac{C_x - S_0}{S_0} \tag{95}$$

Thus, the pH below which precipitation occurs is a function of the amount of salt added initially, the pK_a and the solubility of the free acid formed from the salt.

The analogous equation for salts of weak bases and strong acids (such as pilocarpine hydrochloride, cocaine hydrochloride or codeine phosphate) would be

$$pH_p = pK_a + \log \frac{S_0}{C_x - S_0} \tag{96}$$

in which pK_a refers to the protonated form of the weak base.

Example—Below what pH will free phenobarbital begin to precipitate from a solution initially containing 1.3 g of sodium phenobarbital/100 mL at 25°? The molar solubility of phenobarbital is 0.0050 and its pK_a is 7.41. The molecular weight of sodium phenobarbital is 254.

The molar concentration of salt initially added is

$$C_x = \frac{g/L}{\text{mol wt}} = \frac{13}{254} = 0.051\ M$$

$$pH_p = 7.41 + \log \frac{0.051 - 0.005}{0.005}$$

$$= 7.41 + 0.96 = 8.37$$

Example—Above what pH will free cocaine begin to precipitate from a solution initially containing 0.0294 mole of cocaine hydroethloride/L? The pK_a of cocaine is 5.59, and its molar solubility is 5.60×10^{-3}.

$$pK_a = pK_w - pK_b = 14.00 - 5.59 = 8.41$$

$$pH_p = 8.41 + \log \frac{0.0056}{0.0294 - 0.0056}$$

$$= 8.41 + (-0.63) = 7.78$$

Drug Stability

One of the most diversified and fruitful areas of study is the investigation of the effect of hydrogen-ion concentration on the stability or, in more general terms, the reactivity of pharmaceutical systems. The evidence for enhanced stability of systems when these are maintained within a narrow range of pH, as well as of progressively decreasing stability as the pH departs from the optimum range, is abundant. Stability (or instability) of a system may result from gain or loss of a proton (hydrogen ion) by a substrate molecule—often accompanied by an electronic rearrangement—which reduces (or increases) the reactivity of the molecule. Instability results when the substance desired to remain unchanged is converted to one or more other, unwanted, substances. In aqueous solution, instability may arise through the catalytic effect of acids or bases, the former by transferring a proton to the substrate molecule, the latter by accepting a proton.

Specific illustrations of the effect of hydrogen-ion concentration on the stability of medicinals are myriad; only a few will be given here, these being chosen to show the importance of pH adjustment of solutions that require sterilization.

Morphine solutions are not decomposed during a 60-minute exposure at a temperature of 100° if the pH is less than 5.5; neutral and alkaline solutions, however, are highly unstable. Minimum hydrolytic decomposition of solutions of cocaine occurs in the range of pH of 2 to 5; in one study a solution of cocaine hydrochloride, initially at a pH of 5.7, remained stable during 2 months (although the pH dropped to 4.2 in this time), while another solution buffered to about pH 6 underwent approximately 30% hydrolysis in the same time. Similarly, solutions of procaine hydrochloride containing some hydrochloric acid showed no appreciable decomposition; when dissolved in water alone, 5% of the procaine hydrochloride hydrolyzed, while when buffered to pH 6.5, from 19 to 35% underwent decomposition by hydrolysis. Solutions of thiamine hydrochloride may be sterilized by autoclaving without appreciable decomposition if the pH is below 5; above this thiamine hydrochloride is unstable.

The stability of many disperse systems, and especially of certain emulsions, is often pH-dependent. Information concerning specific emulsion systems, and the effect of pH upon them, may be found in Chapter 20.

Drug Activity

Drugs that are weak acids or weak bases, and hence may exist in ionized or nonionized form (or a mixture of both), may be active in one form but not in the other; often such drugs have an optimum pH range for maximum activity. Thus, mandelic acid, benzoic acid or salicylic acid have pronounced antibacterial activity in nonionized form but have practically no such activity in ionized form. Accordingly, these substances require an acid environment to function effectively as antibacterial agents. For example, sodium benzoate is effective as a preservative in 4% concentration at pH 7.0, in 0.06 to 0.1% concentration at pH 3.5 to 4.0 and in 0.02 to 0.03% concentration at pH 2.3 to 2.4. Other antibacterial agents—on the other hand, are active principally, if not entirely, in cationic form. Included in this category are the acridines and quaternary ammonium compounds.

Drug Absorption

The degree of ionization and lipoid solubility of a drug are two important factors that determine the rate of absorption of drugs from the gastrointestinal tract and, indeed, their passage through cellular membranes generally. Drugs which are weak organic acids or bases, and which in nonionized form are soluble in lipids, apparently are absorbed through cellular membranes by virtue of the lipoidal nature of the membranes. Completely ionized drugs, on the other hand, are absorbed poorly, if at all. Rates of absorption of a variety of drugs are related to their ionization constants and in many cases may be predicted quantitatively on the basis of this relationship. Thus, not only the degree of the acidic or basic character of a drug but consequently also the pH of the physiological medium (gastric or intestinal fluid, plasma, cerebrospinal fluid, etc) in which a drug is dissolved or dispersed—since this pH determines the extent to which the drug will be converted to ionic or nonionic form—become important parameters of drug absorption. Further information on drug absorption is given in Chapter 41.

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Denbigh K: *The Principles of Chemical Equilibrium,* 4th ed, Cambridge Univ Press, 1981.

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Harned HS, Owen BB: *The Physical Chemistry of Electrolytic Solutions,* Reinhold, New York, 1958.

EXHIBIT 4

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Markus HECKER & Andreas H. WAGNER	Group Art Unit: 1632
Serial No.: 10/526,430	Examiner: D. Montanari
Filed: March 1, 2005	Atty. Dkt. No.: DEBE:052US/SLH
For: PHARMACEUTICAL FORMULATION WITH NONSTEROIDAL ANTIPHLOGISTICS AND NUCLEIC ACIDS FOR TRANSFERRING NUCLEIC ACIDS INTO EUKARYOTIC CELLS	Confirmation No.: 9671

CERTIFICATE OF ELECTRONIC SUBMISSION

DATE OF SUBMISSION: October 16, 2008

DECLARATION OF GERHARD BURCKHARDT UNDER 37 C.F.R. §1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

I, Prof. Dr. Gerhard Burckhardt, do declare that:

1. I am a citizen of Germany residing at Göttingen. I currently hold the position of Full Professor and Chairman at the Institute of Physiology of the Georg-August-University Göttingen. My research experience includes well over 120 original articles in peer reviewed international scientific journals and close to 22 review articles in scientific

journals, journal supplements, conference proceedings and books. I have been trained in Medicine and hold university degrees including a doctorate with distinction in Medicine (Dr. med.). I have worked in basic research for almost 35 years, mainly focusing on transport of organic anions across cell membranes. I have a special expertise in renal excretion mechanisms for anionic drugs. A copy of my *curriculum vitae* is attached.

2. Optimization of the pH of a solution containing decoy oligonucleotides (dODNs) or DNA is critical for the delivery of these compounds to the interior of the target cell. Presently, it is not known at a molecular level how dODNs and DNA cross cellular membranes. A simple diffusion can be firmly excluded because of the high water solubility that is due to the presence of numerous negative charges in these molecules. Therefore, it remains that dODNs and DNA are taken up into cells by anion channels, a transporter of the solute carrier family (SLC family), and/or by endocytosis.

3. Anion channels are – in my opinion – unlikely candidates for dODN or DNA uptake. Given the inside negative membrane potential in the range of -40 to -70 mV, these channels would facilitate the efflux of the highly negatively charged dODN or DNA rather than providing an entry pathway against the membrane potential.

4. Among the transporters, SLC19A1, the reduced folate carrier-1 (RFC1 [1]) is a strong candidate for dODN or DNA uptake because this uptake can be competed out by folate. This transporter that is expressed in virtually all tissues of the human body, has a relatively low affinity for folate, and a high affinity for the antineoplastic methotrexate. It

is believed that folate uptake by RFC1 occurs together with the uptake of protons (or the release of hydroxyl ions). An acidification of the extracellular solution (= increase of proton concentration or decrease of hydroxyl ion concentration) would enhance the activity of RFC1. The adjustment of pH is, therefore, critical for the function of RFC1, and would similarly be important to RFC1-mediated uptake of dODN or DNA. Moreover, the pH dependence of RFC may differ between cells / tissues and depend on the phosphorylation status of the transporter [2]. It appears that in many cells the optimal folate transport activity by RFC1 is at pH 7.4, which is higher than observed by the present inventors in their studies. This would argue against a major contribution of RFC1 to the uptake of dODNs and DNA at low pH, but does not exclude a major involvement of RFC1 in uptake at physiological pH (7.4).

5. Besides RFC1, folate receptors (FR) may be responsible for dODN and DNA transport. FR may be involved in folate delivery to target cells [3,4]. These receptors bind folate with very high affinity and are endocytosed together with folate ("cargo"). After acidification of the endosomes and dissociation of receptors and folate, the cargo is released into the cytosol, possibly by anion transporters, and the FR is recycled back to the membrane. Binding of folate to its receptor is enhanced at acidic pH, and so is delivery of folate into RF-expressing cells [5]. Again, adjusting the pH of the solution is critical for the activity of the uptake process through FR. In addition, the release of folate from endosomes into the cytosol is pH-dependent, being faster at acidic pH.

6. It is known that a number of molecules can be taken up through the FR, if coupled to folate. If, as predicted, dODNs or DNA are taken up by FR-induced endocytosis, an acidic pH will enhance uptake, as was in fact observed by the present inventors. Moreover, it is possible that the pH dependence is different for different cargo molecules (dODN; DNA) and, thus, needs to be adjusted individually dependent on the physicochemical properties of the cargo.

7. In conclusion, results of the present inventors suggest that, at a pH of 6.4, dODNs and DNA are preferentially taken up into target cells by binding to the folate receptor and subsequent endocytosis. At a physiological pH of 7.4, uptake of dODNs and DNA may occur mainly through RFC1. Besides pH, structural differences of dODNs and DNA will influence the affinity for binding and translocation by both mechanisms and impact on the relative contribution of RFC1 and FR to overall uptake. The adjustment of pH therefore involves elaborate studies on the uptake of each individual dODN or DNA into model cells as a function of extracellular pH. If uptake cannot be measured directly, e.g. due to the lack of radiolabeled probes, the pH dependence of the cellular effects elicited by dODNs or DNA can serve as a readout. While further experiments will clarify, at a molecular level, the specifics of the transport system for dODNs and DNA, it is already clear that the adjustment of pH in this system is neither trivial nor mere optimization of a routine aspect.

8. I declare that all statements made herein of my own knowledge are true, and that all statements of my own belief are believed to be true, and further that these statements

were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under § 1001 of title 18 of the United States Code.

October 16, 2008
Date

Prof. Dr. Gerhard Burckhardt

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[1] Ganapathy V, Smith SB, Prasad PD. Pflügers Arch – Eur J Physiol 447:641-646, 2004.

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[5] Sierra EE, Brigle KE, Spinelly MJ, Goldman ID. Biochem Pharamcol 50:1287-1294, 1995.

Curriculum vitae

Name	Gerhard Burckhardt
Date and Place of Birth	August 1, 1947, Kaiserslautern (Germany)
Nationality	German
Marrital Status	Married with PD Dr. Birgitta Christina Burckhardt
	Daughter: Friederike Sofie Burckhardt, born June 16, 1982
Academic Education	Medicine, Johann Wolfgang Goethe-Universität, Frankfurt am Main, 1966-1972 *Staatsexamen* (Final Examination) August 9, 1972 ("Very Good")
Promotion	*Dr. med.*, March 29, 1974 Title of Thesis: Determination of K^+ Transport Sites in Ehrlich Mouse Ascites Tumor Cells ("summa cum laude")
Habilitation	Habilitation for *Physiology*, February 4, 1988 Title of Habilitation Thesis: Na^+-H^+ exchange and ATP-driven H^+ Transport in Proximal Tubules of Mammalian Kidney *Privatdozent* (Permission to Teach), May 19, 1988
Professional Career	*Studiendekan* (Subdean for Student Affairs) since April 2008 *Universitätsprofessor* und Direktor (Full Professor and Chair) of the Department Vegetative Physiologie und Pathophysiologie, Georg-August-Universität Göttingen, September 1991 - *Wissenschaftlicher Angestellter* (Research Assistant) at the Max-Planck-Institut für Biophysik, Frankfurt am Main; Department of Physiology (Director: Prof. Dr. med. K. J. Ullrich), September 1978 to August 1991 *Wissenschaftlicher Angestellter* at the Gustav-Embden-Zentrum der Biologischen Chemie (Department of Biochemistry), September 1973 to August 1978 *Medizinalassistent* (Internship), September 1972 to August 1973 *Approbation als Arzt* (Licence) September 3, 1973
Awards, Grants, special functions	Spokesman and Director of the Graduiertenkolleg (PhD Program) 335 "Clinical, Cellular, and Molecular Biology of Internal Organs", 1997 to 2006 President of the German Physiological Society, 2005 Member of the Deutsche Akademie der Naturforscher Leopoldina since 2002 Member of the SFB (Collaborative Research Center) 402 "Molekulare und zellulärc Hepatogastroenterologie" (Spokesman Prof. Dr. K. Jungermann), Göttingen, 1994 to 2000 Member of the SFB 169 "Struktur und Funktion membranständiger Proteine (Spokesman: Prof. Dr. Dr. H. Fasold), Frankfurt, 1984 to 1991 *Preis des Jahres 1975 zur Förderung des Wissenschaftlichen Nachwuchses* donated by the Paul-Ehrlich-Stiftung (Award for the Doctoral Thesis, 1975)
Organisation of Meetings	84th Annual Meeting of the German Physiological Society, Göttingen, March 2005 (Organizer) Transport Colloquium Rauischholzhausen, Marburg, 1999, 2001, 2003, 2005 (Co-organizer) Göttinger Transporttage since 1999 (annually; Organizer) Membranforum "Identifizierung und Molekulare Charakterisierung von Transportproteinen"; Frankfurt, 1987 (Co-organizer) International Symposium "Epithelial Anion Transport - Hormonal Regulation", Frankfurt/Main, 1985 (Co-organizer)

Göttingen, October 14, 2008